Remarks

Claims 1, 5, 8-11 and 16-20 are now pending in this application. Claim 5 is withdrawn. Claims 16-20 are new. Claims 2-4, 6-7 and 12-15 are cancelled by this or previous amendments.

Support for new claims 16-20 can be found in the originals claims as filed, and specifically for example, claims 1 and 8-11.

Claim 1 has been amended to recite "free fraction of thrombin." Support for this amendment can be found throughout the specification and specifically, for example, claim 1 as originally filed and ¶ [006] of the specification.

Paragraph [0013] of the specification has been amended as recommended by the Examiner to include the generic term for POLYBREEN.

No new matter has been added.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1 and 8-11 stand rejected as lacking of written description and as being indefinite because of the use of the language "thrombin not interacting with AT." Claim 1 has been amended to recite "free fraction of thrombin" in place of "thrombin not interacting with AT." With this amendment, applications submit that the rejections have been traversed.

Rejections Under 35 U.S.C. § 103

Claims 1-2, 4, 6-7, and 11 stand rejected as obvious over Plattner, *et al.* ("Plattner") or Philo, *et al.* ("Philo") in view of Winant *et al.*, Furatu, Morris, *et al.*, and Akhavan-Tafti, *et al.* Applicants respectfully disagree with the rejection.

In previous Office Actions and Responses in this application, there has been significant discussion regarding whether Plattner and Philo, in combination with the secondary references,

suggest the determination of AT in a method using a single reaction vessel and a single sample. While that discussion remains appropriate, Applicant submits herewith the Declaration of inventor Dr. Enno Adema. Dr. Adema explains the distinct and non-obvious differences between the mechanism of the method of determining AT in the present invention and the method of Plattner and Philo.

In particular, Plattner does not teach the measurement of AT in the presence of fast acting inhibitors, such as hirudin. Instead, Platter teaches that that AT is measured in the presence of "other plasma proteins which can also inhibit thrombin." (col. 6, lns. 51-52). None of these proteins are described in Plattner, but one of skill in the art would understand that these proteins are slow inhibitors of thrombin (they inhibit thrombin slower than AT). Such inhibitors have been described by Lebreton de Vonne and Mouray, Int. J. Biochem 1980; 12:479-84. (*See* Ex. B to Declaration of Edema).

Plattner teaches the measurement of AT-III in the presence of heparin as an "entity distinct from the 'progressive antithrombin activity' which is measured in the absence of heparin." (col. 6, lns 52-55) From these two determinations, Plattner states that "one can clearly identify a defect in the anticoagulation system as one associated with AT-III rather than other protein inhibiting mechanisms." (col. 6, lns. 55-56)

The measurement of AT-III activity (measurement with heparin) proceeds at a rate that would make the presence of the other proteins inconsequential in the assay. Indeed Plattner, *et al.* states, "[t]he presence of heparin increases the rate of reaction of AT with such proteases approximately 100-fold, ***making AT the only plasma component involved in this rapid reaction***" (emphasis added).

The present invention is distinctly different from the method described in Plattner because the first reaction ***measures the presence of the inhibitor, not AT, as the only plasma component involved*** in the reaction. As recited in step (a) of claim 1, the conditions in the reaction mixture are such that thrombin essentially does not interact with AT, but interacts with the inhibitor.

Drugs that inhibit thrombin, such as Hidurin, act differently in an AT assay than the slow reacting plasma proteins. Hirudin inhibits thrombin at the same fast rate regardless of the presence of heparin. Therefore, when hirudin is present in a sample, AT ***is not the only component in plasma*** that is involved in the inhibition of thrombin, even in the presence of heparin. Moreover, Plattner does not teach anything about measuring thrombin in the presence of an inhibitor for thrombin under conditions that thrombin does not interact with AT. In Plattner, it is expected that the thrombin interacts with AT in the presence of heparin, and also in the absence of heparin in a "progressive antithrombin assay" that is mentioned in Plattner.

Although it may not be explicitly stated, one can infer that Plattner also teaches measurement of these "other protein inhibiting mechanisms" as follows: two measurements of thrombin are made:

a. with heparin – this reaction proceeds very quickly and all of the AT in the sample can be expected to interact with thrombin. Excess thrombin is measured, and "AT is the only plasma protein involved in this rapid reaction." (col. 6, lns. 36-37).

b. progressive antithrombin activity – in order to determine the inhibition by other plasma proteins, the reaction must proceed longer than it takes for thrombin-AT interaction in the presence of heparin. Excess thrombin is

measured, which can be compared to the measurement of excess thrombin in the presence of heparin.

The antithrombin activity determined under "b" minus the antithrombin activity under "a" equals the antithrombin activity of other protein inhibiting mechanisms. An inhibitor like hirudin would not be recognized as "antithrombin activity of other protein inhibiting mechanisms," rather it would be recognized as AT III. Since hirudin is not AT III, the result obtained by following Plattner is false high.

The Examiner cites Philo for essentially the same proposition as Plattner. Philo teaches assays with and without heparin. In the progressive antithrombin assay of Philo, the reaction proceeds to completion after an incubation time of one hour. As in Plattner, fast acting thrombin inhibitors will affect the result with and without heparin. There is no possibility to distinguish between AT and a fast acting inhibitor and, thus, no possibility to determine the true AT level in the presence of such inhibitors.

Accordingly, neither Plattner nor Philo determine AT-III, in either one reaction vessel or two, as described in the present invention. Using the methods of Plattner and Philo, inhibitors of thrombin would be recognized as AT, which would present falsely high results. The present invention accounts for the inhibitor because the inhibitors interact quickly with thrombin, and the first thrombin activity measurement is taken without AT interaction. Neither Plattner nor Philo describe this mechanism.

Accordingly, one of skill in the art would not be lead to the present invention based upon the teaching of Plattner and Philo. Moreover, the secondary references do not resolve the lack of the teaching of the basic mechanism of the invention. For instance, Furata teaches the determination of GOT first, then the level of GPT, to obtain the level of GPT. This detection of

two different analytes is different than the presently claimed method where only a single analyte (AT) is measured and the interfering substance is not determined. Morris describe a decision tree where, depending on the first result (the antinuclear antibody test), other tests are performed. The tests are performed sequentially and are unrelated, other than the sense of a clinical/diagnostic logic. Akhavan-Tafti concerns the visualization of different analytes on a solid phase through the use of different probes. The claimed assay is homogeneous and uses a single probe (thrombin in combination with its chromogenic substrate). None of these references address the deficiencies of Plattner and Philo and suggest that an accurate determination of AT can be conducted in the presence of an inhibitor as claimed.

New claim 16 has been added to address the Examiner's concern that the method of claim 1 may be conducted on samples that do not contain a thrombin inhibitor. *See* Office Action, p. 14, ¶ 20. Applicants respectfully disagree that this is an issue, since one of the benefits of the invention is that a clinician does not need to know whether or not the sample contains an inhibitor before ordering an analysis of the sample with the current method. The method will provide an accurate results, regardless of the presence of the inhibitor, which is a significant improvement over the prior art. Applicants have amended claim 1 to recite a sample "containing" one or more pharmaceutical compounds that inhibit thrombin. With the amendment, however, it is clear that neither Plattner nor Philo nor any of the secondary references teach the determination of AT in samples containing a pharmaceutical compound that inhibits thrombin.

In addition, the Examiner has suggested that it is necessary for the applicants to prove that the ordinary artisan would have thought the assaying of AT in the presence of the thrombin inhibitors was impossible. Applicants respectfully disagree that this is the standard for establishing or refuting a *prima facie* case of obviousness. In any event, Dr. Adema explains

why one of skill in the art, when considering the prior art method, would have concluded the interfering factor was AT, which would provide a falsely high result. *See* Declaration, ¶¶ 10-11.

Finally, the Examiner has previously dismissed Applicants' evidence of long-felt need because it was not presented in a Declaration. *See* Office Action mailed June 9, 2009, p. 20. Through his Declaration, Dr. Adema now explains that the claimed invention is an alternative to assays that use Factor Xa as the binding partner for ATIII. While Factor Xa is not inhibited by hirudin and similar drugs that directly inhibit thrombin, many new drugs are being developed, some of which are inhibitors of Factor Xa (*e.g.,* Fondaparinux; *see* Ex. C., Braun, *et al.,* (*see* Results and Discussion) and K. Hickey *et al.,* Figure 3, GTH Congress 2008). These substances lead to false high AT result in Xa-based AT assays. Thus, the Xa-based AT assays suffer from the same problem as AT assays using thrombin. The present method is able to detect ATIII regardless of the inhibitor that may be present in the sample. Because the laboratory may which drug that patient may taken prior to a blood sample for use in an AT assay, the use of the claimed invention is advantageous because it would allow for the determination of AT regardless of presence or kind of drug. Braun, *et al.* and Hickey, *et al.* show that the problem of the presence of interfering factors in the determination remains unresolved by current practices. This problem was well known prior to the filing of the application (*see* Ex. 1, hereto, Beeck, *et al.,* Blood Coagulation and Fibrinolysis, 2000, 11:127-135; *see specifically, for example,* p. 131 right column – p. 132.[1] Beeck, *et al.* teaches "As a consequence, in patients under treatment with thrombin inhibitors, plasma AT activity must not be monitored by thrombin-based assays." *Id.,* p. 132. The problem with thrombin assays when thrombin inhibitors are present is resolved by the currently claimed method.

[1] This article is not recited in the Declaration of Dr. Adema, but is found as reference 3 in the bibliography of Braun, *et al.,* (Ex. C to the Declaration).

In view of the foregoing, Applicants respectfully request that the rejection of claims 1-2, 4, 6-7, and 11 under 35 U.S.C. § 103 be withdrawn.

Claims 8 and 9 stand rejected as obvious over Plattner in view of Winant *et al.*, Furatu, Morris, *et al.*, and Akhavan-Tafti, *et al.*, and further in view of Exner (US 6.051,434).

Claims 8 and 9 depend from claim 1, and therefore include all of the limitation so claim 1. Because Exner does not add to the discussion regarding the rejection of claim 1, claims 8 and 9 are patentable for at least the same reasons as claim 1.

Conclusion

Applicants submit that the forgoing amendments are remarks are sufficient to address all of the pending rejections. Applicants, however, do not waive any argument by failing to make the argument here. Applicants expressly reserve the right to reassert the above arguments, or assert additional arguments in the future.

Applicants respectfully contend that all conditions of patentability are met in the pending claims as amended. Allowance of the claims is thereby respectfully solicited.

If the Examiner believes it to be helpful, he is invited to contact the undersigned representative by telephone at 312 913 0001.

Respectfully submitted

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EXHIBIT 1

Blood Coagulation and Fibrinolysis 2000, 11:127–135

Measurement of antithrombin activity by thrombin-based and by factor Xa-based chromogenic substrate assays

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Functionally active antithrombin can be quantified by chromogenic substrate assays utilizing the heparin cofactor activity of antithrombin and the inhibition rates of thrombin or of activated factor X (FXa). Thrombin-based assays but not FXa-based assays may overestimate the antithrombin activity due to their sensitivity toward heparin cofactor II. We focused on the question whether an overestimation of antithrombin activity by thrombin-based assays involves the risk of misdiagnosing antithrombin-deficient individuals as being non-deficient. We determined antithrombin using two thrombin-based assays and one FXa-based assay in 27 plasma samples from patients with acquired antithrombin deficiency spiked with lepirudin, in antithrombin-deficient plasma and in mixtures of antithrombin-deficient plasma and normal plasma. We also measured antithrombin in healthy subjects, in patients with inherited and acquired antithrombin deficiency and in patients under high-dose heparin treatment. At therapeutic final concentrations of lepirudin, antithrombin activities were considerably overestimated by the thrombin-based assays but not by the FXa-based assay. The residual antithrombin activities in antithrombin-deficient plasma determined by the thrombin-based assays were markedly higher than the corresponding values obtained with the FXa-based assay. The thrombin-based assays also overestimated antithrombin activity in patients under high-dose heparin. However, the degree of overestimation in the range between 50 and 100 IU/dl was too low to misidentify individuals with inherited or acquired antithrombin deficiency as normal. We conclude that functionally active antithrombin can be reliably determined using FXa-based chromogenic substrate assays in all settings examined. Thrombin-based assays must not be used in patients under treatment with hirudin or other direct thrombin inhibitors. *Blood Coag Fibrinol* 11:127–135 © 2000 Lippincott Williams & Wilkins.

Keywords: antithrombin, chromogenic substrate assays, specificity, thrombin, factor Xa, lepirudin, antithrombin deficiency, heparin

Introduction

The determination of plasma antithrombin (AT) activity is an important measure to recognize congenital and acquired AT deficiency states possibly requiring therapeutic intervention [1,2] or for predicting poor prognosis [1,3,4]. Functionally active AT is usually quantified by chromogenic substrate assays utilizing its heparin cofactor activity and the inhibition rates of thrombin or activated factor X (FXa) [5–7]. On principle, thrombin-based assays are less specific than FXa-based assays, since heparin cofactor II (HC II) specifically inhibits thrombin, and thus may interfere with thrombin inactivation [8–10]. Actually, comparative measurements of AT activities in AT-deficient patients using an assay based on human thrombin and a FXa-based AT assay have demonstrated that there might be a

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greater possibility of mislabelling an AT-deficient individual as being non-deficient when the thrombin-based assay is used [11].

Specificity of thrombin-based AT assays can be improved by several measures including short incubation times [12], replacement of human thrombin by bovine thrombin [9,13], increase in ionic strength (Dr Arnulf Hubbuch, Roche Diagnostics, Mannheim, Germany, personal communication), and the reduction of heparin concentration in the assay [14]. Recent work has shown that AT activities can obviously still be overestimated by these more specific second-generation thrombin-based assays in patients under high-dose heparin and that this is primarily due to HC II activity [15]. However, the clinical significance of these observations has to be challenged, since it has not been examined whether a marginal overestimation of AT activity may actually result in missing AT deficiency states.

We checked the specificity of two thrombin-based assays and one FXa-based assay by spiking plasma samples from AT-deficient individuals with therapeutic concentrations of the recombinant lepirudin (Refludan™), since direct thrombin inhibitors are increasingly used for antithrombotic treatment [16]. We additionally examined specificity by *in vitro* experiments utilizing AT-deficient plasma and by investigations in healthy individuals, in AT-deficient patients and in patients under high-dose heparin.

Materials and methods

In vitro *experiments*

Lepirudin (Refludan™) was purchased from Hoechst (Frankfurt, Germany). This preparation was added to 27 plasma samples from patients with acquired AT deficiency to receive final concentrations of 0.5, 1.0, 1.5 and 2.0 mg/l, respectively.

Mixing tests were performed using 'Reference Plasma 100%' from Immuno Diagnostics (Heidelberg, Germany) and two AT-deficient plasmas ['AT-deficient plasma I' from LOXO (Heidelberg, Germany) and 'AT-deficient plasma II' from Roche Diagnostics (Mannheim, Germany)]. 'Reference Plasma 100%' is a pool plasma from healthy individuals, which has been calibrated against the International Reference Preparation for Antithrombin III, Plasma, 72/1. The residual AT activities of the AT-deficient plasma samples I and II, measured by the FXa-based AT assay, were 4 and 2 IU/100 ml, respectively. The HC II activities were 104 and 70 IU/100 ml, respectively.

Subjects

We measured AT in 180 healthy blood donors who had not donated blood or plasma within 3 months prior to the study. Fifty-two of these blood donors were male, 78 were females not using oral contraceptives (OC), and the remaining 50 females were using OC. In these three study groups, the mean age was 40, 38 and 29 years, and the age range was 19–67, 20–66 and 19–42 years, respectively. In OC users, the phase of menstrual cycle, kind of preparation and duration of oral contraceptive use was not considered. AT was also quantified in 52 patients with acquired AT deficiency (AT, 75 IU/100 ml or lower, measured by the thrombin-based assay from Roche Diagnostics) and in 31 patients under high-dose unfractionated heparin (UFH). The patients with acquired AT deficiency suffered from liver disease or from disseminated intravascular coagulation and were not under UFH treatment. Patients under high-dose UFH received doses between 150 and 600 IU/kg body weight per 24 h in order to maintain the ratio of the patient's activated partial thromboplastin time to the mean of normal pooled plasma within the range 1.5–2.5. We also examined plasma samples from six patients with hereditary AT deficiency type I. Plasma samples from a further 27 patients with acquired AT deficiency who were not under UFH treatment and had AT levels of 60 IU/dl or lower were used for our *in vitro* experiments examining the influence of lepirudin on the AT assay results.

Blood was drawn from the antecubital vein using 21-gauge butterfly needles. The first 5 ml were discarded. Blood samples were collected into plastic tubes (Sarstedt Nümbrecht, Germany) containing 0.106 mol/l trisodium citrate (nine parts blood, one part trisodium citrate). Samples were centrifuged at 3300 × *g* for 10 min at room temperature within 2 h of collection. Plasma was subsequently separated, and aliquots of 0.5 ml were snap-frozen and stored in plastic tubes at −70°C. At the time of assay, plasma samples were thawed at 37°C for 10 min. All analyses were performed within 60 min after thawing.

Assays

We measured AT activities by two thrombin-based chromogenic substrate assays and by one FXa-based chromogenic substrate assay under comprehensively standardized conditions. The thrombin-based assays were from Roche Diagnostics (AT-R), and from Immuno Heidelberg (AT-I), and the FXa-based assay (Coamate® AT 400) was from Haemochrom

(Essen, Germany) (AT-H). All analyses were performed by one technical assistant using a Hitachi 717 Analyzer from Roche Diagnostics. AT was determined kinetically at 405 nm utilizing one-point calibration. All assays were calibrated against the International Reference Preparation for Antithrombin III, Plasma, 72/1, which had been purchased from the National Institute for Biological Standards and Control. For the *in vitro* experiments, calibration was performed utilizing 'Reference Plasma 100%'. Plasma samples were not prediluted. The two thrombin-based assays mainly differed in their final heparin concentration and the chromogenic substrate used to measure the remaining bovine thrombin activity. The final heparin concentrations of AT-R, AT-I and AT-H were 1.65, 1.24 and 2.6 IU/ml, respectively.

Intra-assay and interassay imprecisions were obtained by 15- or 20-fold determination of AT activities in 'normal' and 'abnormal' control plasmas, respectively. The control plasma utilized was a plasma pool from 30 healthy individuals. This plasma was used undiluted and at two dilutions using Owren's Veronal buffer to receive calculated values of 75 and 50 IU/dl AT activities.

We determined AT antigen on a Dade Behring Nephelometer analyzer by kinetic nephelometry using reagents from Dade Behring (Liederbach, Germany). The International Reference Preparation 72/1 was used for calibration. We quantified HC II activity by a chromogenic substrate assay from Stago (Asnières, France).

Statistical analysis

The values obtained were analyzed by the Kolmogoroff–Smirnoff test for normal distribution. It was confirmed that the distributions of AT in the different subgroups were approximately normal. Therefore, these values are given as means and standard deviations (SD). One-way analysis of variance and unpaired t test were used for comparisons of mean values in the various groups. Mean values in samples from the same patient were compared by the paired t test, with $P < 0.05$ indicating statistical significance. For comparison of assays, the subgroups of individuals not receiving heparin and of patients under high-dose heparin were also examined. Since the distribution of these data was skewed, the Wilxocon signed-rank test was used. Correlation between parameters was performed by calculating Pearson's rank correlation coefficient, and $P < 0.01$ indicated statistical significance. Agreement between methods was established by analyzing the data according to the graphic method described by Bland and Altman [17,18]. The AT assays were arbitrarily judged to be interchangeable if the limits of agreement [mean difference (bias) ± 2 SD] did not exceed 10 IU/dl.

Results

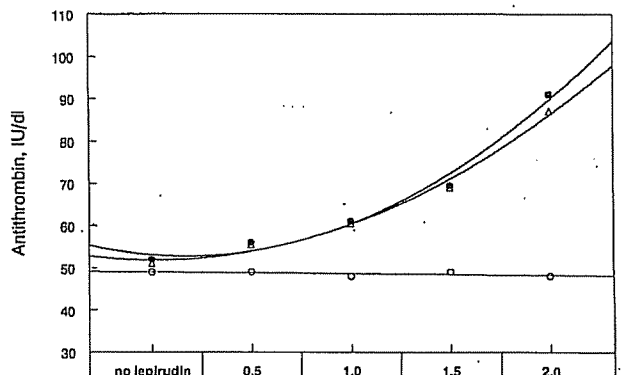
Reproducibility

Measurements of intra-assay and interassay imprecisions, considering all three assays and all three measuring ranges, resulted in variation coefficients (CVs) between 1.2 and 2.4% and between 2.5 and 4.7%, respectively. At 50 IU/dl, the CVs for AT-H, AT-I and AT-R were 2.6, 3.6 and 4.7%, respectively.

In vitro *investigations*

The addition of lepirudin to 27 plasma samples from patients with acquired AT deficiency resulted in a dose-dependent increase of AT activities determined by the thrombin-based assays, whereas the AT-H assay results were not influenced (Fig. 1). A final concentration of 2.0 mg/l lepirudin caused a mean overestimation of the AT activities quantified by AT-R and AT-I of 39 and 43%, respectively.

The AT activities measured in the two AT-deficient plasma samples and in mixtures of AT-deficient plasma and pool plasma ('Reference Plasma 100%') are shown in Figure 2. The residual activities determined in the two AT-deficient plasma samples



	no lepirudin	0.5	1.0	1.5	2.0
● AT-R	52	56	61	69.5	91
△ AT-I	51	55.5	60.5	69	87
○ AT-H	49	49	48	49	48

Figure 1. Mean antithrombin (AT) activities measured in 27 plasma samples from AT-deficient patients using the two thrombin-based assays AT-R and AT-I, and the activated factor X-based assay AT-H. The plasma samples were spiked with lepirudin to achieve final concentrations of 0.5, 1.0, 1.5 and 2.0 mg/l, respectively.

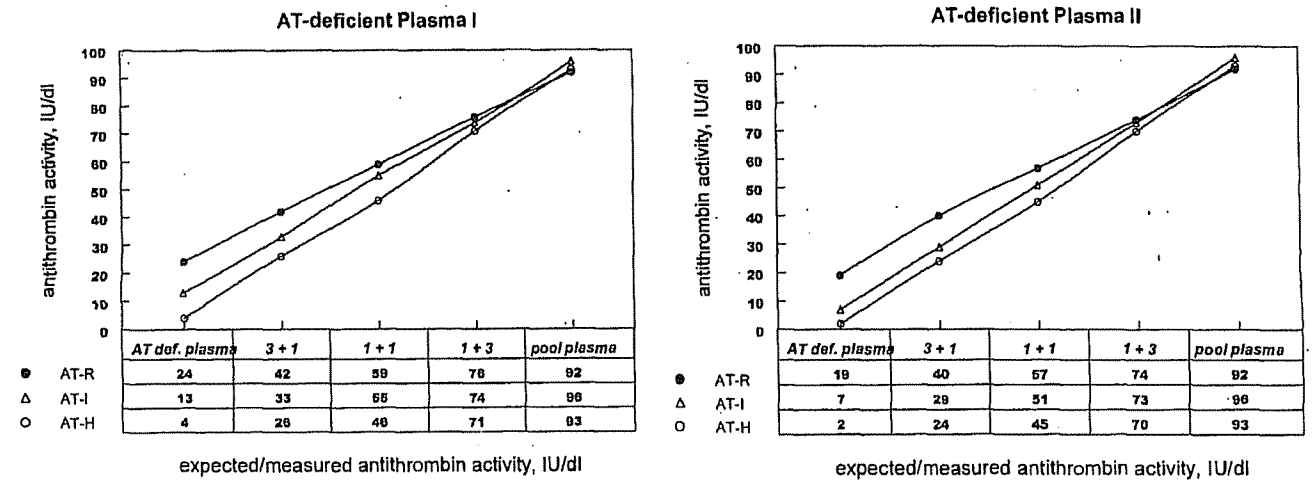


Figure 2. Antithrombin (AT) activities determined in two AT-deficient plasma samples, in pool plasma and in mixtures of AT-deficient plasma and pool plasma, using the two thrombin-based assays AT-R and AT-I, and the activated factor X-based assay AT-H.

by AT-H were 4 and 2 IU/dl, respectively. The two thrombin-based assays yielded substantially greater AT activities (AT-R, 24 and 19 IU/dl; AT-I, 13 and 7 IU/dl). However, in mixtures of AT-deficient plasma and pool plasma suggesting expected AT activities of 50 and 75 IU/dl, respectively, these discrepancies were substantially lower. Considering the respective reference ranges for the three assays, unspecificity of the thrombin-based assays did not result in borderline or normal AT activities measured in these specimens.

AT activities in different study groups

The results are presented in Table 1. In all study groups, AT-R yielded significantly higher AT activities than AT-I and the FXa-based assay AT-H ($P < 0.0001$). In all 232 subjects not receiving heparin (180 healthy individuals, 52 patients with acquired AT deficiency), there was a close linear correlation between the AT activities obtained by the three different methods (Fig. 3). Despite identical calibration procedures and close linear correlations, however, the limits of agreement between AT-R and AT-I, and between AT-R and AT-H clearly exceeded the chosen acceptable difference of 10 IU/dl (Fig. 3). Hence, these methods were judged not to be interchangeable in these settings. In contrast, AT-I and AT-H were found to be interchangeable. Figure 3 also demonstrates that the lack of agreement between AT-R and the remaining AT assays was mainly due to AT values being within the respective reference ranges. This observation is supported by the results exclusively obtained from patients with acquired AT deficiency. Figure 4 shows that there was both a close linear correlation and an acceptable difference between the AT activities measured by the three different assays. These findings suggest that non-heparinized AT-deficient individuals are not misidentified as normal, based on the results obtained from thrombin-based assays.

Table 1. Antithronbim (AT) measured by two thrombin-based assays (AT-R, AT-I) and by an activated factor X-based assay (AT-H) in different study groups

Study group	*n*	AT-R	AT-I	AT-H
I. Healthy men	52	105 ± 8*	98 ± 7*	99 ± 8^{NS}
II. Healthy females, OC non-users	78	107 ± 8*	98 ± 8*	96 ± 9*
III. Healthy females, OC users	50	101 ± 10*	93 ± 9*	89 ± 10*
IV. Acquired AT deficiency	52	60 ± 8*	56 ± 8*	57 ± 8**
V. High-dose heparin	31	87 ± 14*	83 ± 14*	76 ± 14*

AT-R, AT-I, and AT-H indicate AT activities (IU/dl) measured by the three different assays. Data are mean ± SD. OC, oral contraceptive.
*$P < 0.0001$, AT-R versus AT-I, AT-R versus AT-H and AT-I versus AT-H. **$P < 0.001$, AT-I versus AT-H. ^{NS}, Not significant, AT-I versus AT-H in healthy men.

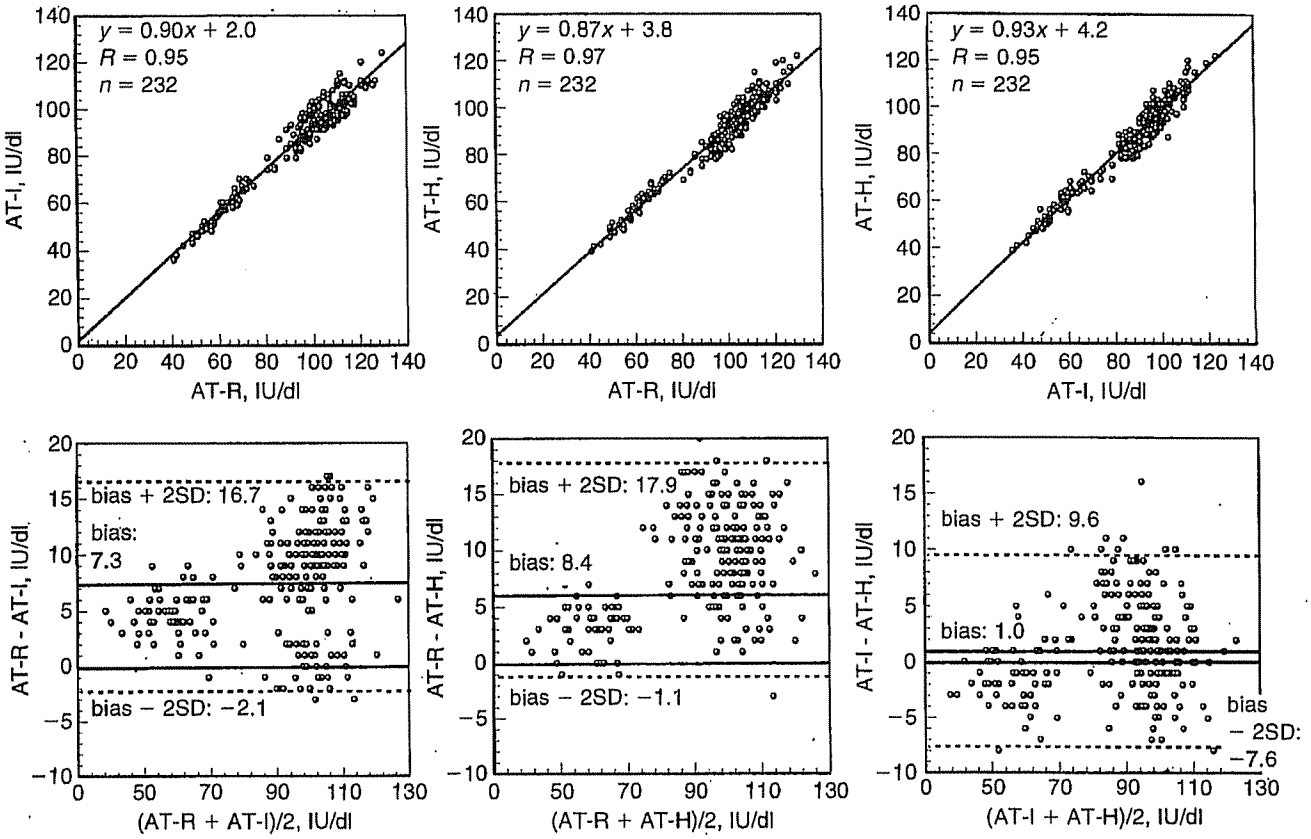


Figure 3. Correlations between antithrombin (AT) activities determined by two thrombin-based assays (AT-R and AT-I) and by an activated factor X-based assay (AT-H), and comparison of methods (Bland–Altman plot) in healthy individuals and in non-heparinized patients with acquired AT deficiency. The dashed lines indicate the limits of agreement (± 2SD). The bias indicates the mean difference between methods; $n = 232$.

We also found all AT activities well below the lower limit of the respective reference range in six patients with inherited AT deficiency type I (Table 2).

In the 31 patients under high-dose heparin, AT activities measured by the two thrombin-based assays were substantially higher than the values obtained with the anti-FXa assay, with mean differences (biases) of 10.9 and 6.2 IU/dl, respectively (Fig. 5). However, there was also a marked bias of 4.6 IU/dl between the two thrombin-based assays.

OC users had significantly lower AT levels than OC non-users (assay AT-R, $P = 0.012$; assay AT-I, $P = 0.001$; assay AT-H, $P = 0.003$) and than men (assay AT-R, $P = 0.020$; assay AT-I, $P = 0.001$; assay AT-H, $P < 0.0001$). Since there were no significant differences between men and OC non-users, both groups were integrated into one group for establishing a reference range ($n = 130$). The lower limits of the reference range (mean − 2SD) determined by AT-I and AT-H were 82 and 79 IU/dl, respectively, and agreed well with the results reported by others [19,20]. However, the lower limit of the reference range established by AT-R was 90 IU/dl.

Discussion

Previous work has demonstrated that thrombin-based AT assays but not FXa-based assays overestimate the plasma AT activities due to their sensitivity towards HC II [10,11,15] and thus involve the risk of misdiagnosing AT-deficient individuals as being non-deficient. We reassessed the specificity of two second-generation thrombin-based assays compared with an AT-FXa assay in different settings. We attached importance to comprehensively standardized conditions including identical calibration procedures for the three assays.

The addition of the hirudin analogue lepirudin to plasma samples from patients with acquired AT deficiency resulted in a dose-dependent increase of AT activities determined by the thrombin-based assays, whereas the AT-H assay results were not

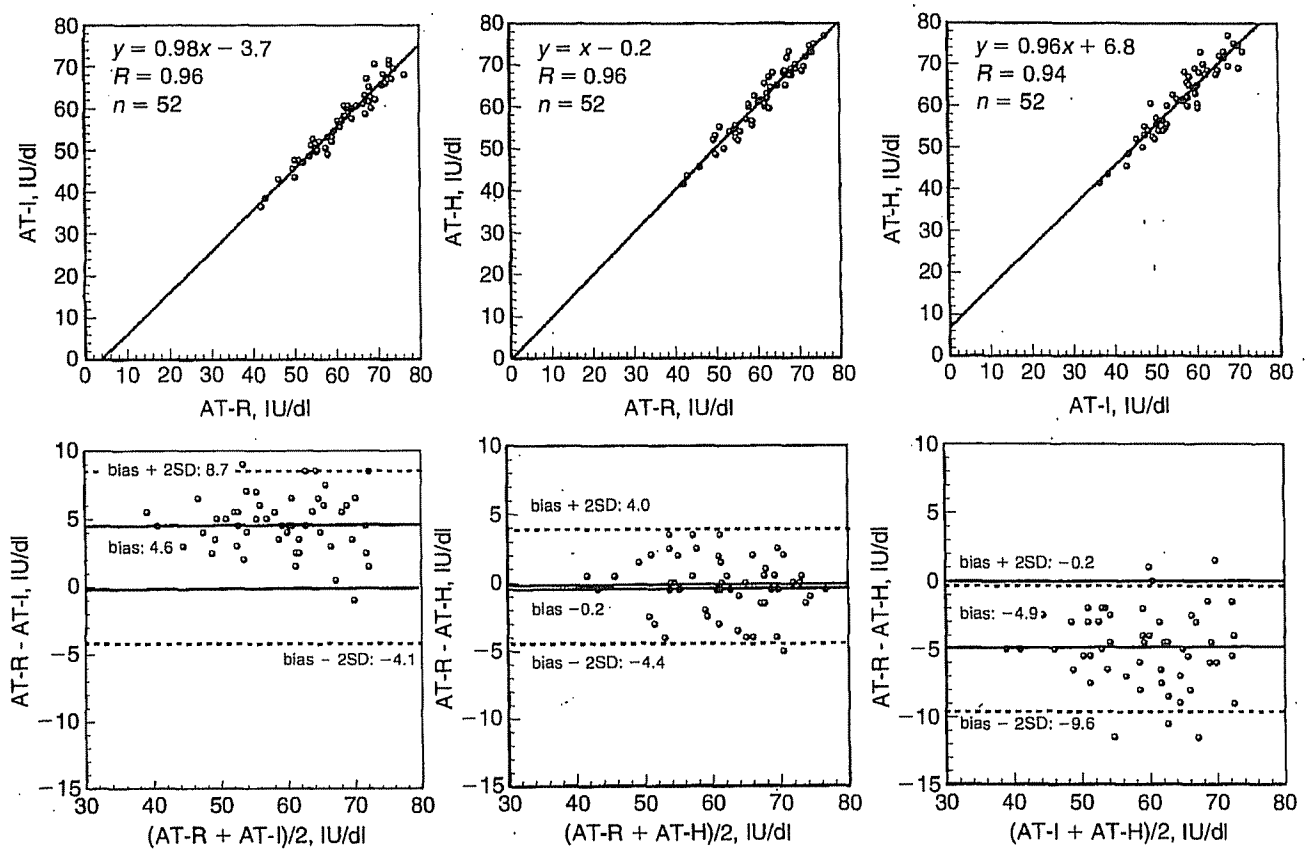


Figure 4. Correlations between antithrombin (AT) activities determined by two thrombin-based assays (AT-R and AT-I) and by an activated factor X-based assay (AT-H), and comparison of methods (Bland–Altman plot) in non-heparinized patients with acquired AT deficiency. The dashed lines indicate the limits of agreement (± 2SD). The bias indicates the mean difference between methods; $n = 52$.

Table 2. Plasma antithrombin (AT) antigen, antithrombin activities measured by two thrombin-based assays (AT-R and AT-I) and an activated factor X-based assay (AT-H), and plasma heparin cofactor II activity (HC II) in six patients with congenital antithrombin deficiency type I (IU/dl)

Patient	AT antigen	AT-R	AT-I	AT-H	HC II
1	49	47	57	43	100
2	57	59	57	55	95
3	55	58	65	50	120
4	40	49	55	37	125
5	57	60	68	53	122
6	55	54	51	54	100

influenced (Fig. 1). The final concentrations of lepirudin were comparable with the plasma levels observed in patients under treatment [21–23]. We could demonstrate that the direct dose-dependent inhibition of the bovine thrombin utilized in the assays by hirudin or its analogues may actually lead to excessive overestimation of AT activity and misdiagnosing AT-deficient patients as being non-deficient. As a consequence, in patients under treatment with thrombin inhibitors, plasma AT activity must not be monitored by thrombin-based assays.

Impaired specificity of thrombin-based AT assays is supported by our *in vitro* investigations using two different AT-depleted plasma samples containing normal HC II activities. The purpose of these studies was to simulate congential AT deficiency by

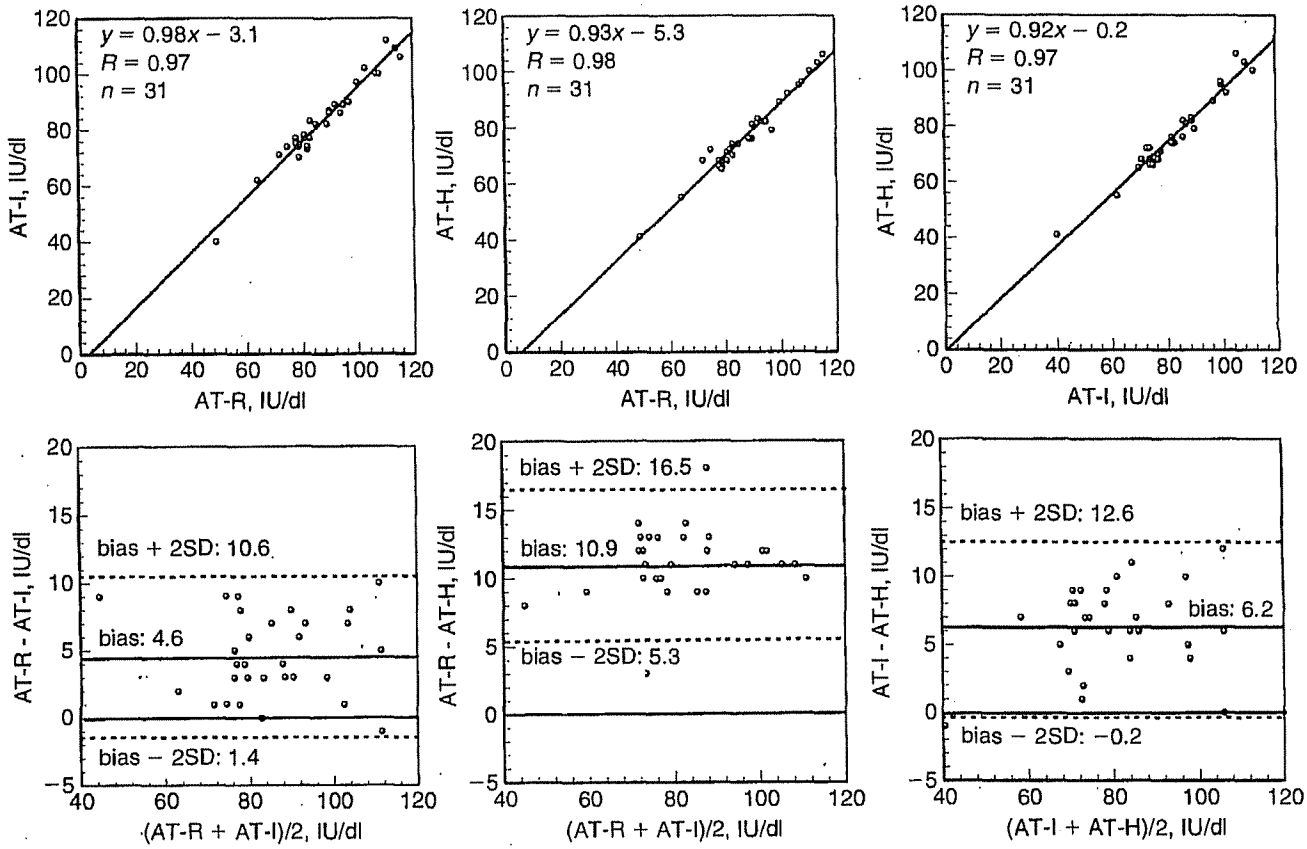


Figure 5. Correlations between antithrombin (AT) activities determined by two thrombin-based assays (AT-R and AT-I) and by an activated factor X-based assay (AT-H), and comparison of methods (Bland–Altman plot) in patients under high-dose unfractionated heparin. The dashed lines indicate the limits of agreement (± 2SD). The bias indicates the mean difference between methods; $n = 31$.

mixing tests using AT-deficient plasma and normal plasma. These mixtures provide the same matrix as expected in congential AT-deficient individuals. We found markedly higher residual AT activities with AT-R and AT-I compared with the FXa-based assay. However, in mixtures of AT-deficient plasma and normal pool plasma suggesting expected AT activities of 50 and 75 IU/dl, the discrepancies between the thrombin-based assay results and the values obtained with the AT-FXa assay were substantially lower than in the pure AT-deficient plasma samples (Fig. 2). This may be explained by the fact that AT-deficient plasma contains normal levels of functionally active HC II and that the residual AT activity measured by thrombin-based assays is mainly due to HC II activity. The percentage of AT activity caused by HC II decreases with increasing amounts of AT in the mixtures of AT-deficient plasma and normal plasma. These data confirm previous findings that thrombin-based assays overestimate the AT activities in plasma due to their sensitivity toward HC II [10–12,15]. However, the impaired specificity of thrombin-based assays obviously does not result in mislabelling of non-heparinized AT-deficient patients as being non-deficient.

In healthy individuals and in patients with acquired AT deficiency not receiving heparin, there were only negligible differences between the results obtained with the thrombin-based assay AT-I and the FXa-based assay (Table 1 and Fig. 3). Measurement of AT by AT-R resulted in a more marked overestimation. It could be demonstrated that the AT-R results and the corresponding data obtained from the assays AT-I and AT-H could not be used interchangeably in these settings. However, in patients presenting acquired AT deficiency, there were only marginal differences between the AT plasma levels obtained with all three assays (Fig. 4). The observed degrees of overestimation appeared to be too low to misidentify acquired AT-deficient individuals. These findings were supported by the results obtained in six individuals with congenital AT deficiency type I. All three AT activity assays and the AT antigen assay yielded AT plasma levels clearly below the lower limit of the respective

reference range (Table 2). We could not include more congenital AT-deficient subjects in our study, since these conditions are rare, even among patients with thrombosis [24].

In contrast, the two second-generation thrombin-based assays overestimated plasma AT activity in patients under high-dose UFH (Fig. 5). These findings agreed with recent observations by another study group [15], indicating the contribution of HC II/heparin complexes to antithrombin activity measured by thrombin-based assays. However, the substantial differences between the results obtained with the two thrombin-based assays demonstrate that the specificity of these assays strongly depends on the assay conditions. It was shown by others that the specificity of thrombin-based AT assays can be improved by reducing the final heparin concentration [14]. This heparin concentration was lower in the AT-I assay than in the AT-R assay.

We could confirm former findings that OC users had significantly lower plasma AT activities than OC non-users and males, and that there were no significant differences between OC non-users and males [25,26].

The lower limit of the reference range for AT-R was substantially higher than the values obtained with AT-I and AT-H, and those reported by other study groups [19,20]. Since we used identical calibration procedures and identical equipment for all three assays, these findings suggest that each laboratory should establish specific reference ranges for different AT activity assays.

In summary, plasma AT activity can be reliably measured using second-generation thrombin-based assays in individuals receiving no heparin. In patients under high-dose UFH, thrombin-based assays may yield higher AT levels than FXa-based assays due to their sensitivity toward HC II. It remains to be determined whether AT-deficient patients simultaneously receiving high-dose UFH may be misdiagnosed as normal, as a consequence of thrombin-based AT assay results. In patients receiving hirudin or other direct thrombin inhibitors, AT assays based on the inhibition of thrombin must not be used, since AT levels can be substantially overestimated, resulting in a large number of missing AT deficiency states. FXa-based AT assays yield reliable results in all settings examined in our study.

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